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Searching The Hidden Potential of Actinomycetes Prevalent In Nagpur Soil As Future Antibiotic Producers

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Abstract:-
In the present scenario, ever-increasing antibiotic drug resistance with human pathogens commonly been reported. In requirement, development of new antibiotics also remains the thrust area. The present study attempted to isolate antibiotic producing actinomycetes from soil surface which finds commonplace in the ability to control human pathogen. The study detected *Amycolatopsis equina, Amycolatopsis keratiniphila* and *Amycolatopsis speibonae* able to provide bioactive agent by which uro-pathogens like *Pseudomonas sp* and *Salmonella species* prominently get inhibited by the partially purified antibiotic like molecule.
In a conclusive remark, actinomycetes of Nagpur soil able to produce many bioactive compounds which find commonplace in controlling MDR as well as sensitive uro-pathogens mainly *Pseudomonas* species.

Keywords: - Antibiotics, Actinomycetes, Multidrug resistance control; soil sampling.

Introduction

With the largest taxonomic unit, phylum Actinobacteria stands vital in a bacterial domain (Ludwig et al., 2012). Actinobacteria remain prevalent in the soil as well as in aquatic environment as free-living organisms (Macagnan et al., 2006). They survive in harsh conditions as saprophytic soil-dwelling organisms even under limited nutrient conditions (Mayfield et al., 1972). They are prevalent in alkaline and organic-rich matter soil also and prevalent till the depth of 2 m below ground (Good fellow and Williams, 1983). In a soil, dominant actinomycetes recorded as *Streptomyces* accounting close to 94% (Williams and Vickers, 1988). These Streptomyces showcase this dominance since they were able to grow under acidic soil also (pH 3.5) (Kim et al., 2003).
These actinomycetes (mainly Streptomyces) able to produce two-third of antibiotics and finds promises to control multidrug-resistant pathogens (Bennett, 1998; Ilic et al., 2007).
They are capable of this feature since they live in competitive mode in soil, and hence the timing of development purely depends on molecules they produce (Colson et al., 2007).
In a view, number of Actinobacteria reported to produce bioactive agents such as *Verrucosispora sp* (Abyssomycin) (Bister et al., 2014; *Streptomyces canus* (Amphomycin) (Heinemann et al; 1953; *Micromonospora sp*. (Anthracyclin) (Grein et al., 1980); *Streptomyces canus* (Aspartocins) (Yang et al., 2014); and *Marinispora sp* (Marinomycin) (Kwon et al, 2006).
In the present study, a similar attempt been made to isolate Actinomycetes from Nagpur soil. These were then tested to produce antibiotics, and purified antibiotic will be tested against

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uro-pathogens grouped as Multidrug resistance (MDR) and non MDR category. Finally, identity of promising actinomycetes will bedone by 16S rRNA gene sequencing.

Method

A) Soil sampling

In the present study, antibiotic-producing actinomycetes attempted to isolate from the Nagpur agricultural soils. In a requirement, five soil samples by the depth of 25 cm from the soil surface collected from the selected region which appeared with black colour soil in texture. The soil sampling mainly been done from rhizosphere region and sampled in a sterile plastic bag.

B) Processing of soil sample

Collected soil sieved through 250 μm pore size and fine soil weighed as 1 gram added to 10 ml physiological saline (NaCl, 8.5 g/ L) after that preparation homogenized by shaking in vortex mixture. Soil suspension (1 ml) was then added to 9 ml sterile saline and mixed to homogeneity. The sample then diluted up to 10^{-5} concentration using standard dilution method and one ml of each diluent used as an inoculum.

C) Inoculation of specific media

One ml soil diluent has directly inoculated on the starch casein agar (SCA) which is specific for the actinomycetes growth. Here SCA composition given as starch 10 g; casein (vitamin free) 0.30 g; KNO_3 2.00 g; $MgSO_4.7H_2O$ 0.05g; K_2HPO_4 2.00g; NaCl 2.00g; $FeSO_4.H_2O$ 0.01 g, $CaCO_3$ 0.02 g and Agar 18.00 gm per litre with pH set at 7.0 ± 0.1. Once inoculated, SCA was incubated at 37 °C for 48 hours to record the presence of colonies. This medium remains supplemented with amoxicillin (20 μg/ml) and cycloheximide (25 μg/ ml) that inhibits bacteria and fungi to grow, respectively.

D) Morphology study of Actinomycetes

Confirmation of actinomycetes recorded with white, cream, grey, orange and other colour colonies. These colonies observed under dissecting microscope and compound microscope to record aerial mycelia, colony and growth form, soluble pigment and spore forms to distinguish actinomycetes as per morphology.

Primary screening of Antibacterial activity.

The ability for antibiotic production has been assessed by co-culturing the human pathogens as a target organism with actinomycetes able to produce antibiotics using cross inoculation method.

In a nutrient agar medium, Actinomycetes inoculated broth previously stressed up to 45°C for expression of antibiotic used as inoculum on it in a straight-line format. Upon straight line inoculation, perpendicular straight-line inoculum made with pathogens (*E. coli, P. aeruginosa, K. pneumoniae, S. aureus,* and *S. typhi*) giving the appearance as of plus sign.

Given type of co-culturing, creates an opportunity to mass screen actinomycetes against number of human pathogens. All plates then incubated at 37 °C for 24 hrs to confirm the ability of growth inhibition by actinomycetes once growth inhibition recorded close to the growth of actinomycetes.

E) Secondary screening

The antibiotic-producing actinomycetes able to control uro-pathogens studied in primary screening further checked for their *in vitro* ability to produce antibiotics once grown in a starch casein broth (200 ml). Hereupon incubation obtained growth centrifuged, and supernatant filtered sterilised. It was then precipitated by taking 100 ml of it and added with dichloromethane: ethyl acetate and n- butanol (50 ml in 1:1:1 ratio). Further by mixing,

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obtained organic solvent extract allowed to get evaporated and obtained dried fraction once again dissolved in sterile distilled water and checked for antibacterial assay by a well diffusion method having 100 µl volume.

F) Actinomycetes Identification by 16S rRNA

Identified potential Actinomycetes were targeted for their 16s rRNA by using the protocol suggested by Rai et al., (2013) using universal primers and sequencer.

Result

Soil sample and sampling of actinomycetes

Nagpur city agricultural soil sampled at five locations recorded with 37 isolates of Actinomycetes once allowed to grow on SCA medium once inoculated with a diluted concentration of soil (Table 1). The growth of these isolates showcased with confluency on the SCA medium as given in Fig 1.

Morphology of Actinomycetes

These thirty-seven actinomycetes recorded for their Aerial mycelia, Growth and colony form, soluble pigment and spore forms as given in Table 2.

Primary screening of Antibacterial Assay.

These thirty-seven isolates checked for their ability to control co-cultured *E. coli, S. aureus, Klebsiella sp., Pseudomonas sp.* and *Salmonella sp.* As per the overall study, it has been observed that *E. coli, S. aureus* and *Klebsiella species* did not get growth inhibited with all actinomycetes tested. In contrast, *Salmonella species* recorded to get inhibited by three actinomycetes and seven strains of Actinomycetes species growth inhibited *Pseudomonas* successfully indicated that *Pseudomonas species* remains the most sensitive species with these actinomycetes (Table 3) (Fig 2).

Secondary screening

The solvent extracted antibiotic like compound once tested by well diffusion method against *Pseudomonas species* and four actinomycetes registered better growth controlling activity against non–MDR *Pseudomonas* species as compared to MDR *Pseudomonas species* as in Table 4.

Identification of Actinomycetes

Once the ability of four actinomycetes understood to control *Pseudomonas* species; their identity confirmed by 16S rRNA gene sequencing. As per sequencing, isolate 16 confirmed as *Amycolatopsisequina strain SE(8)3;* isolate 19 as *Amycolatopsis equina;* isolate 20as *Amycolatopsis keratiniphila* and isolate 21as *Amycolatopsis speibonae*
as in Fig 3-6.

Discussion

In the present study, soil harbouring actinomycetes investigated in detail for their ability to control Uropathogens (*E. coli, S. aureus, Pseudomonas sp.* and *Salmonella sp.*) and many of their strains found to be capable of producing antibiotics successfully that remains specific in its action.

Soil found to be a prominent source of antibiotic-producing actinomycetes reported as early as in 1965; which remain affected by the present pH and acidic conditions of soil in the region. (Williams et al., 1971). Similar to the present study Indian soil also been reported to be actinomycetes antibiotic-producing (n=20) that controls *E. coli* and *S. aureus* profoundly (Rai et al., 2016). Hasnaa et al., (2018) recorded Rabat neighbourhood soil able to record 17.24% pigment-producing actinomycetes found to be antimicrobial producer in nature.

In the present study, isolation of Actinomycetes made realistic on SCA medium. In a similar study, soil of Koringa mangrove of Andrapradesh reported being positive for actinomycetes with ability to express gelatinase, chitinase, α- amylase, protease and urease.

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Shah et al., (2017) isolated Actinomycetes from soils of Kashmir Himalayas mainly *Streptomyces pratensis* able to control *S. aureus,* and *Mycobacterium tuberculosis* once detected on SCA medium.

In the present study as per primary assay selected Actinomycetes when co-cultured with uro-pathogens, they failed to inhibit *E. coli, S. aureus* and *Klebsiella species* but able to *Pseudomonas sp.* and *Salmonella sp.* successfully.

Similarly Anansiriwattana et al., (2006) by carrying out mass screening of Actinomycetes put forward bacteria *Streptomyces, Micromonospora* and Nocardia able to control pathogens *P. aeruginosa*, *E. coli*, *B. subtilis* and other; since they confirm to form Geldanamycin successfully. Srivibool and Sukchotiratana (2006) confirmed that actinomycetes isolated from coastal soils able to be antimicrobial since they can control *Streptomyces sp.* and *Actiomadura sp*.

In the present study, four actinomycetes belong to *Amycolatopsis* species identified as *Amycolatopsis equina* (n= 2), *Amycolatopisis keratiniphila* (n=1) and *Amycolaptosis speibonae* (n=1). The ability of *Amycolaptosis sp.* already been found to be holding potential for antibiotic production since *A. circi* sp. no, *A. equina* and*A. hippodormi* (Everest and Meyers (2011); *A. balhimycina sp. nov., A. tolypomycina sp. nov; A. vancoresmycina* and *A. keratinophila sub sp. Keratiniphila sub. sp. nov* (Wink et al., 2003).

Conclusion

Human started to explore nature with the modern methodologies but remain stick to the fundamental part like soil to begin its journey towards success. In the present study also to combat ever-increasing disease incidences and antibiotic resistance found to be promising to control by searching new antibiotic producers from soil. We represent that soil harbours many potential actinomycetes capable of producing antibiotics and by which better growth controlling ability against Uropathogens finds new hopes. The study put forward that Actinomycetes of *Amycolatopsis species* are more prominent in antibiotic production and also remains more prevalent in Nagpur soil as investigated.

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Table 1: Actinomycetes in number recorded on SCA medium from five soil samples of Nagpur region

Site	1	2	3	4	5
Actinomycetes	5	10	15	03	4
Total :37 Isolates					

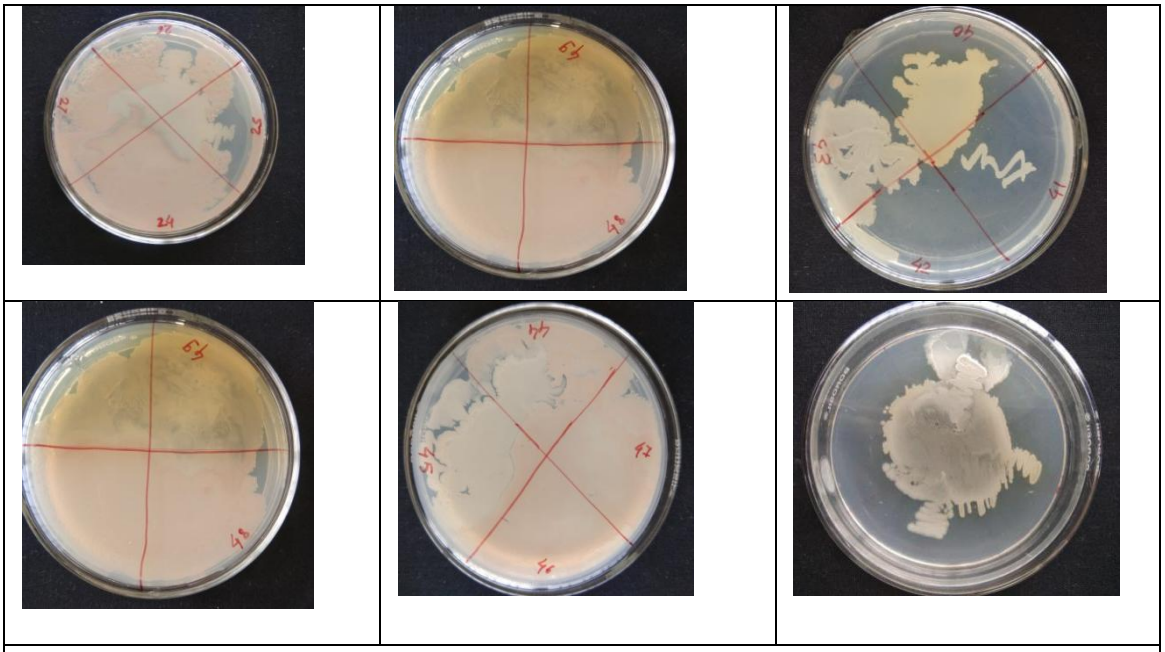

Fig. 1: Adapted Actinomycetes able to grow on the SCA medium under *in vitro* conditions.

Table 3: Different features of colonies spores and pigments recorded with all actinomycetes isolated from the soil region of Nagpur

Isolates	S1a	S1b	S1c	S1d	S1e
Aerial mycelia	Pale-brown	Gray	White	White	Dark-Gray
Growth and colony form	Abundant and rhizoid	Moderate, complex, tough	Moderate, Oval, tough and leathery	Abundant,	Abundant, rhizoid, and leathery

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Soluble pigment	Yellow pigment	Dark-gray pigment	None	Yellow pigment	Dark brown pigment
Spore forms	Chainlike in rectiflexous form	Oval spores in spiral chains	Ornamented in open primitivespiral	Smooth and round in spiral	Oval spores in spiral chains

Table 3 cont··· 2 of 7

Isolates	S2a	S2b	S2c	S2d	S2e
Aerial mycelia	Pale-brown	White	Dark-Gray	White	Dark-Gray
Growth and colony form	Abundant and rhizoid	Moderate, Oval, tough and leathery	Abundant, rhizoid, and leathery	Abundant,	Abundant, rhizoid, and leathery
Soluble pigment	Yellow pigment	None	Dark brown pigment	Yellow pigment	Dark brown pigment
Spore forms	Chainlike in rectiflexous form	Ornamented in open primitivespiral	Oval spores in spiral chains	Smooth and round in spiral	Oval spores in spiral chains

Table 3 contd····· 1 of 7

Table 3 cont···4 of 7

Table 3 cont···4 of 7

Isolates	S3a	S3b	S3c	S3d	S3e
Aerial mycelia	Gray	Pale-brown	Dark-Gray	Dark-Gray	White
Growth and colony form	Moderate, Oval, tough and leathery	Abundant and rhizoid	Moderate, complex, and leathery	Abundant, rhizoid, and leathery	Moderate, Oval, tough and leathery
Soluble pigment	None	Yellow pigment	Dark-gray pigment	Dark brown pigment	None
Spore forms	Oval spores in spiral chains	Chainlike in rectiflexous form	Oval spores in spiral chains	Oval spores in spiral chains	Smooth and round in spiral
	Ornamented in open primitivespiral				Ornamented in open primitivespiral

Table 3 cont··· 5 of 7

Isolates	S5a	S5b	S5c	S5d	S5e
Aerial mycelia	Gray	Pale-brown	Pale-brown	White	Gray
Growth and colony form	Moderate, complex, tough	Abundant and rhizoid	Abundant and rhizoid	Moderate, Oval, tough and leathery	Moderate, complex, tough

Soluble pigment	Dark-gray pigment	Yellow pigment	Yellow pigment	None	Dark-gray pigment
Spore forms	Oval spores in spiral chains	Chainlike in rectiflexous form	Chainlike in rectiflexous form	Ornamented in open primitivespiral	Oval spores in spiral chains

Table 3 cont⋯ 6 of 7					
Isolates	S6a	S6b	S6c	S6d	S6e
Aerial mycelia	White	Pale-brown	Gray	White	Gray
Growth and colony form	Moderate, Oval, tough and leathery	Abundant and rhizoid	Moderate, complex, tough	Abundant,	Moderate, complex, tough
Soluble pigment	None	Yellow pigment	Dark-gray pigment	Yellow pigment	Dark-gray pigment
Spore forms	Ornamented in open primitive spiral	Chainlike in rectiflexous form	Oval spores in spiral chains	Smooth and round in spiral	Oval spores in spiral chains

Table 3 cont⋯ 7 of 7					
Isolates	S7a	S7b	S7c	S7d	S7e
Aerial mycelia	White	Pale-brown	Dark-Gray	White	Pale-brown
Growth and colony form	Moderate, Oval, tough and leathery	Abundant and rhizoid	Abundant, rhizoid, and leathery	Abundant,	Abundant and rhizoid
Soluble pigment	None	Yellow pigment	Dark brown pigment	Yellow pigment	Yellow pigment
Spore forms	Ornamented in open primitive spiral	Chainlike in rectiflexous form	Oval spores in spiral chains	Smooth and round in spiral	Chainlike in rectiflexous form

Table 3:- Co-cultured uropathogens growth inhibited by the soil actinomycetes as per primary screening.

Sr. No.	*E. coli*	*S. aureus*	*Pseudomonas sp.*	*Klebsiella sp.*	*Salmonella sp.*
1	x	x	x	x	x
2	x	x	x	x	x
3	x	x	x	x	x
4	x	x	x	x	x
5	x	x	x	x	x
6	x	x	x	x	x
7	x	x	x	x	x
8	x	x	x	x	Inhibited
9	x	x	x	x	x

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10	x	x	x	x	x
11	x	x	x	x	x
12	x	x	x	x	x
13	x	x	x	x	x
14	x	x	x	x	x
15	x	x	x	x	x
16	x	x	Inhibited	x	x
17	x	x	x	x	x
18	x	x	x	x	x
19	x	x	Inhibited	x	x
20	x	x	Inhibited	x	x
21	x	x	Inhibited	x	x
22	x	x	x	x	x
23	x	x	x	x	x
24	x	x	x	x	x
25	x	x	Inhibited	x	Inhibited
26	x	x	x	x	x
27	x	x	Inhibited	x	Inhibited
28	x	x	x	x	x
29	x	x	x	x	x
30	x	x	x	x	x
31	x	x	x	x	x
32	x	x	x	x	x
33	x	x	x	x	x
34	x	x	x	x	x
35	x	x	x	x	x
36	x	x	x	x	x
37	x	x	Inhibited	x	x

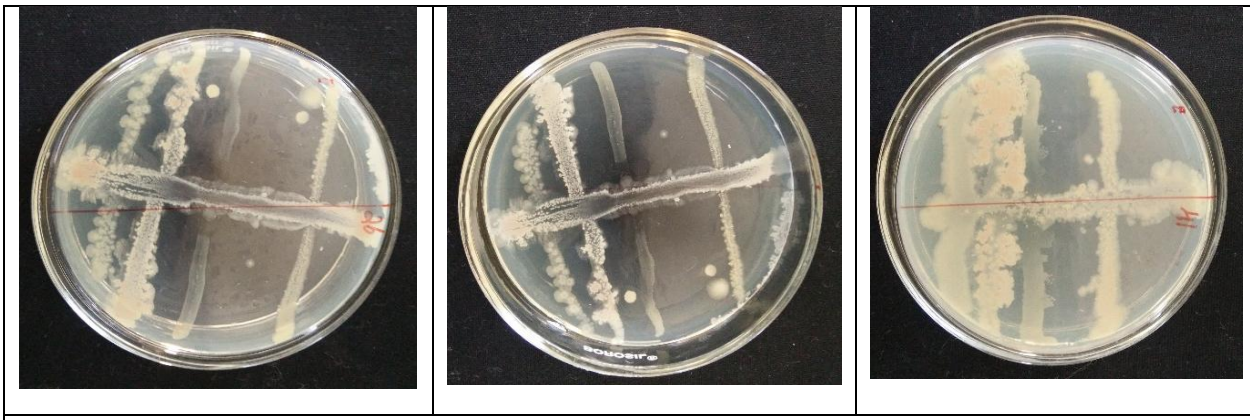

Fig. 2: Co-cultured actinomycetes and pathogens indicated that actinomycetes able to produce antibiotic like molecules since pathogen growth inhibition recorded around actinomycetes colonies.

Table 4:- Actinomycetes based partially purified antibiotic like molecules able to control MDR and non MDR *Pseudomonassp.* under *in vitro* conditions.

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Actinomycetes	MDR zone of inhibition in mm					Non-MDR zone of inhibition in mm					MDR Avg ± std. dev	Non - MDR	P-value
	1	2	3	4	5	1	2	3	4	5			
16	12	18	19	12	15	16	17	20	21	22	15 ±3	19±2.5	0.0644 ns
19	10	10	11	15	18	14	12	13	15	16	12.80 ± 3.56	14.00 ± 1.58	0.5180
20	18	17	08	13	12	14	14	12	09	09	13.60 ± 4.03	11.60 ± 2.5	0.3744
21	08	07	06	08	08	12	12	13	12	09	7.4 ± 0.89	11.60 ± 1.5	0.0007

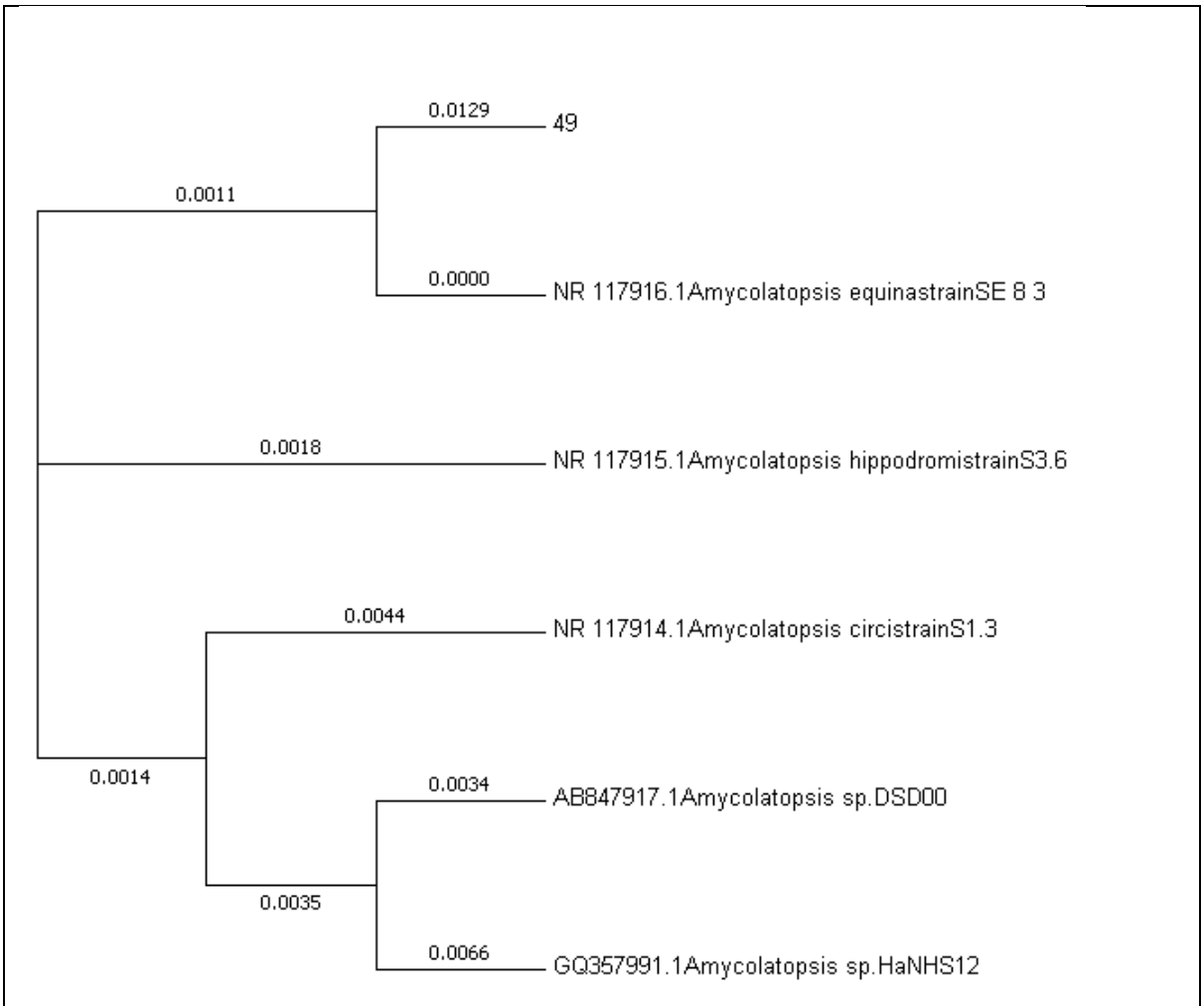

Fig. 3: Isolate 16(49) registered best homology with *Amycolatopsis equina* as per homology when sequenced for the 16S rRNA gene.

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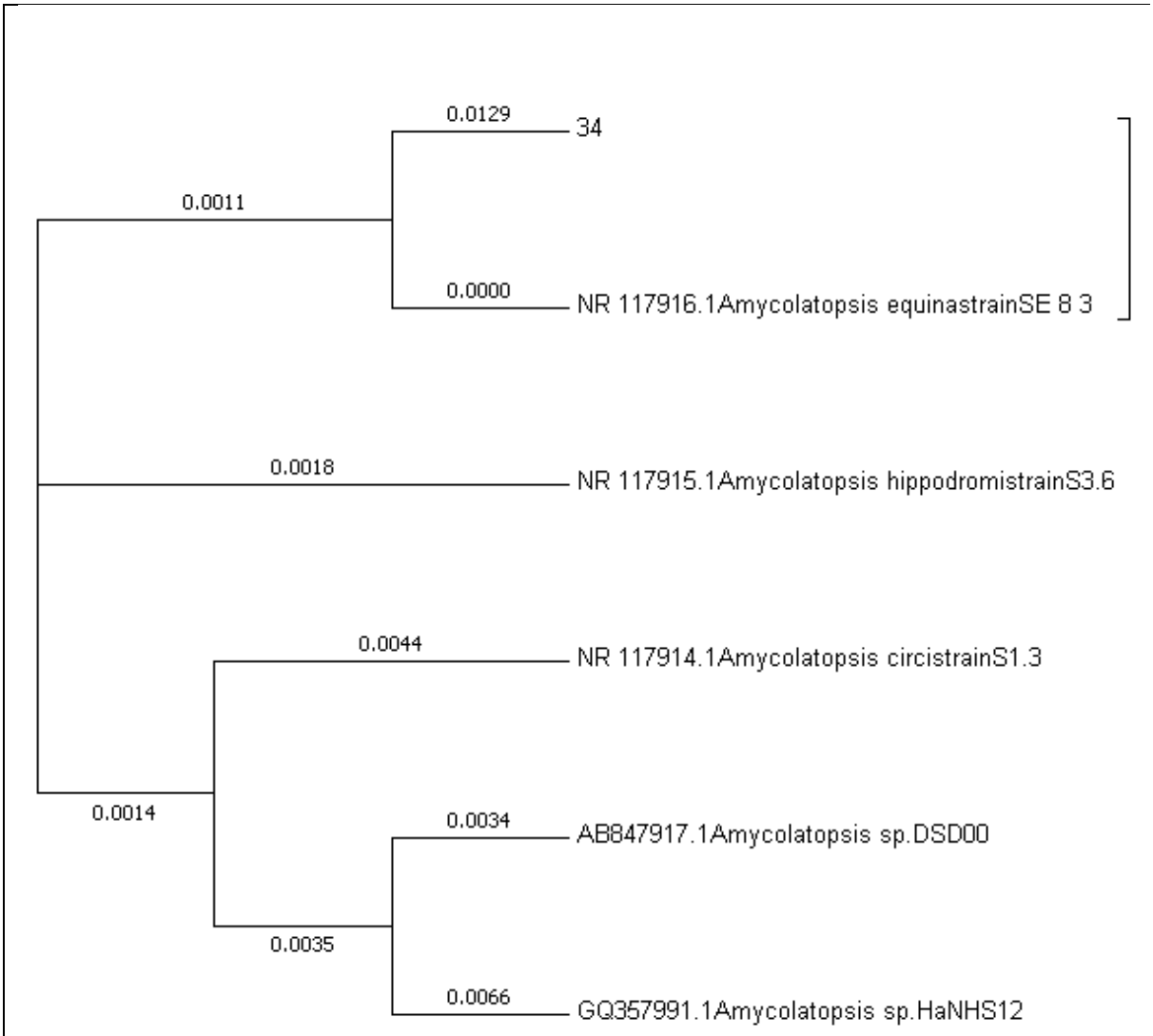

Fig. 4: Isolate 19(34) registered best homology with *Amycolatopsis equina* as per homology when sequenced for the 16S rRNA gene.

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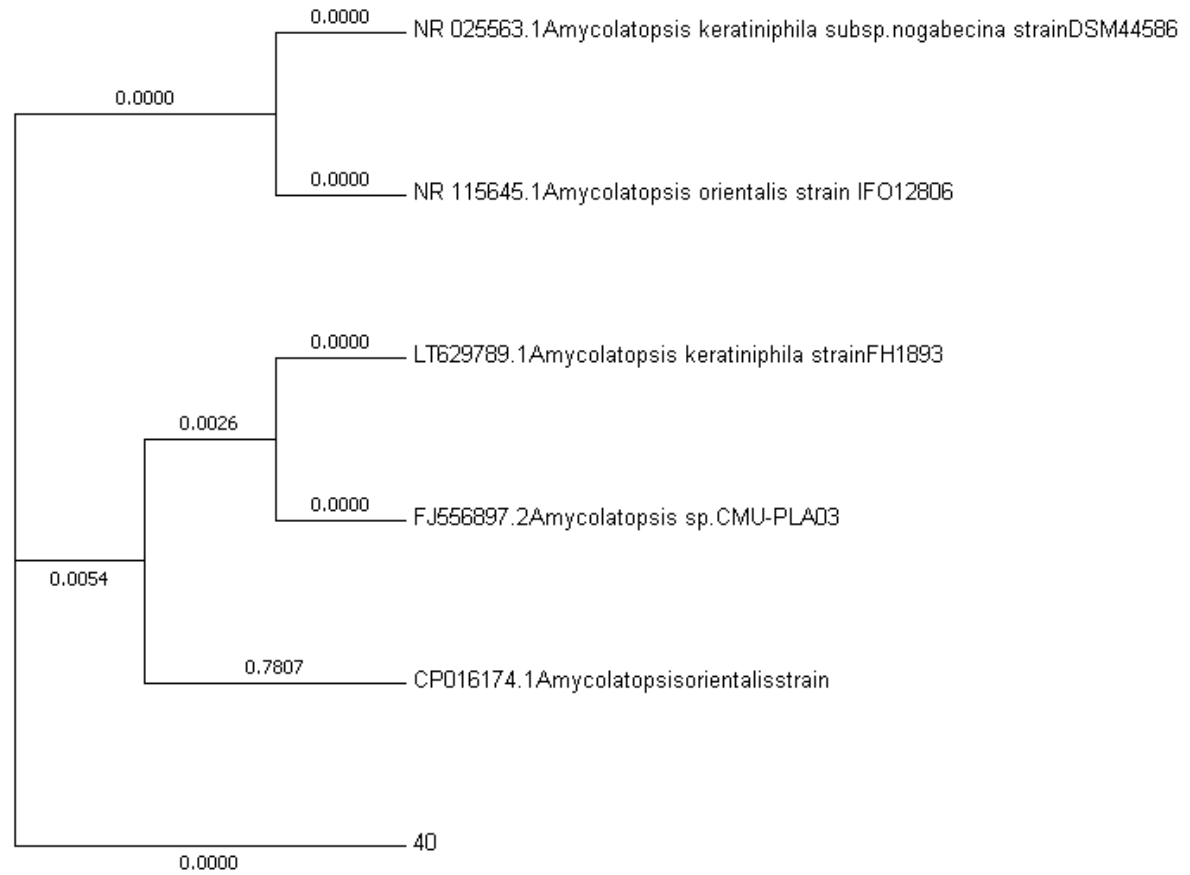

Fig. 5: Isolate 20(40) registered best homology with *Amycolatopsis keratiniphila* as per homology when sequenced for the 16S rRNA gene.

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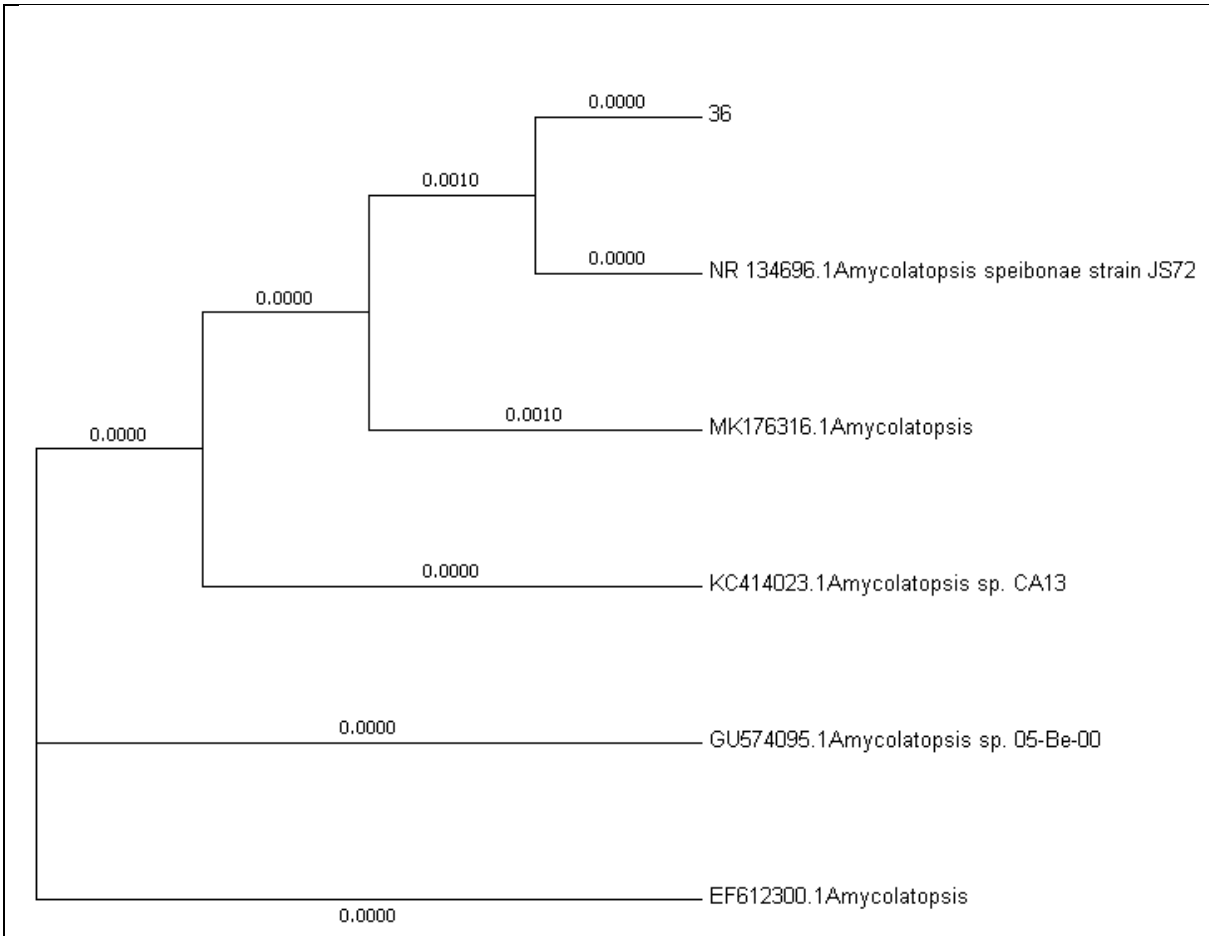

Fig. 6: Isolate 21(36) registered best homology with *Amycolatopsis speibonae* as per homology when sequenced for the 16S rRNA gene.